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13. ABSTRACT *(Maximum 200 words)*

The purpose of this work is to generate neutralizing human monoclonal antibodies to Botulinum neurotoxins (BoNT) A, B, and E. To generate a large panel of antibodies, mice transgenic for the human immunoglobulin locus were immunized with BoNT A, B; and E binding domain (H_C). RNA was prepared, the human variable regions amplified by PCR and used to construct human single chain Fv (scFv) antibody fragment gene repertoires. The repertoires were cloned to create phage display libraries. Selection of the libraries on BoNT/A, B, and E H_C results in the isolation of a large panel of human monoclonal scFv antibody fragments. To demonstrate in vivo toxin neutralization, it was necessary to express the scFv as fusions with the human IgG1 Fc region from the yeast Pichia pastoris. This resulted in a significant increase in the serum half life in vivo. Previously isolated murine scFv expresed as scFv-Fc fusions showed toxin neutralization in vivo, with a combination of two scFv-Fc fusions able to neutralize 100 toxin LD50s. Ongoing work includes: 1) efforts to further increase the serum half life of scFv-Fc fusions to increase potency and the ability to identify the most potent neutralizing scFv; and 2) further characterization of the recently isolated human scFv. Ultimately neutralizing scFv will be used to construct completely human neutralizing IgG. This offers a route to immunoprotective passive immunotherapy for prevention and treatment of Botulinum neurotoxin intoxication.

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5. Introduction

5.1. The problem to be studied

The overall goal of this proposal is to produce neutralizing human monoclonal antibodies against Botulinum neurotoxins for immunoprophylaxis and immunotherapy. Antibodies will be generated using a novel approach, phage display, which overcomes the limitations of conventional hybridoma technology. The proposal represents a continuation of work begun under DAMD17-94-C-4034 titled "Production of Human Antibodies which Neutralize Botulinum Neurotoxin Type A". In the sections below, we first describe the problem, the limitations of currently available reagents, the novel approach we have used for this work (phage display), results obtained to date under DAMD17-94-C-4034, and specific aims and experimental design for the current preproposal.

5.1.1. Background

Botulinum neurotoxins (BoNT) are neurotoxic proteins that block the release of the neurotransmitter acetylcholine leading to flaccid paralysis and death from asphyxia. Inadvertent ingestion of contaminated food leads to the clinical disease Botulism. BoNTs have also been intentionally produced for use as biological warfare agents and weapons of terror due to their potency (microgram quantities are lethal) and ease of production using unsophisticated techniques For example, Iraq has declared the production of at least 19,000 liters of concentrated BoNT, of which 10,000 liters were loaded into munitions (1). Consequently, countermeasures designed to protect against this biological warfare agent have been developed, but each one has limitations. A polyvalent vaccine has been developed, but protective immunity takes months to develop and may be directed against only one or two of the seven distinct serotypes. Furthermore, vaccination requires identification of the population at risk. This is straightforward for battlefield troops, but impossible for civilians who could be exposed if the agent was used as a weapon of terror. Widespread immunization is also becoming less attractive since BoNTs are increasingly being used as a therapy for human diseases (2). Vaccination would deprive individuals of subsequent toxin therapy. Alternatively, neutralizing antibodies could be administered prophylactically or therapeutically to the population at risk. Polyvalent equine or human immune globulin protect experimental animals (3) and appear to protect humans (4) against BoNT intoxication. Immune globulin is most effective when administered prior to exposure, but can prevent disease up to 24 hours post exposure depending on the dose and route of exposure (3). With supportive care, immunoglobulin therapy reduces the duration of illness and cost of hospitalization. These studies demonstrate the efficacy of passive immunotherapy, however equine immune globulin has a high incidence of side effects, including serum sickness and anaphylaxis (5). Human immune globulin should prove nontoxic, but requires a source of immunized human plasma donors.

Neutralizing monoclonal antibodies would provide an unlimited amount of antibody of defined specificity and reproducible titer, but to date no efficacious neutralizing monoclonal antibodies have been produced despite "years of effort by several fine laboratories" (3). Potential reasons for this failure include: 1) rarity of B-cells producing neutralizing antibodies in the polyclonal response elicited by available immunogens; 2) Inadequate binding affinity of monoclonals produced to date for neutralization of a toxin with a high affinity (K_d ~ 1 nM) for its receptor; or 3) the need

to block several epitopes on the toxin for neutralization. Furthermore, murine monoclonal antibodies are not ideal therapeutics since they are immunogenic when administered to humans, resulting in decreased efficacy over time and the risk of allergic reactions. Thus the ideal therapeutic would be neutralizing human monoclonal antibodies. In general, however, human monoclonal antibodies have proven extremely difficult to make using conventional hybridoma technology (6) and are frequently IgM and of low affinity.

5.1.2. A novel approach for production of monoclonal antibodies

To overcome the limitations of conventional hybridoma technology, we and others have developed technology which permits generation of recombinant monoclonal antibodies in E. coli (reviewed in (7-9). This approach has proven possible due to three technical achievements. First, the antigen binding V_H and V_L domains of antibodies can be expressed in E. coli, either as Fab fragments or as single chain Fv (scFv) fragments. Second, large and diverse repertoires of Fab or scFv genes can be generated using the polymerase chain reaction (PCR). Third, the scFv or Fab antibody fragments can be expressed on the surface of viruses (phage) that infect E. coli. The resulting phage has the antibody fragment on its surface, anchored to the phage via the coat protein, and contains the gene encoding the antibody inside the phage. Thus the phage mimics the function of the B-lymphocyte, providing a physical linkage between phenotype on the surface and genotype within.

scFv or Fab gene repertoires can be cloned into phage vectors, resulting in the creation of phage antibody libraries. Phage antibodies binding a specific antigen can be separated from non-binding phage by selection on antigen. Phage are incubated with immobilized antigen, non-binding phage removed by washing, and bound phage eluted. A single round of selection will result in a 20 to 1000 fold enrichment for binding phage. Eluted phage are used to infect E. coli, which produce more phage for the next round of selection. Repetition of the selection process makes it possible to isolate binding phage present at frequencies of less than 1 in a billion.

This technology can be applied in a number of ways to produce monoclonal antibodies (10-12). First, it can be used to bypass conventional hybridoma technology. After murine or human immunization, B-lymphocytes are isolated from spleen, bone marrow, or peripheral blood, and the V-genes used to create phage antibody libraries. Binding antibody fragments are isolated by selection on antigen. This approach yields a greater number of antibodies than hybridoma technology, especially for human antibodies. In addition, the V-genes are already cloned and the antibody fragments typically express well in E. coli. This facilitates subsequent genetic engineering, such as creation of diagnostic or therapeutic fusion proteins, or *in vitro* affinity maturation. For therapeutic use, the antibody genes can be subcloned into eukaryotic expression vectors for expression of complete IgG antibodies (13).

Alternatively, it is possible to bypass immunization entirely to produce human antibodies (14, 15). The high transformation efficiency of E. coli allows creation of very large (10^9 to 10^{10} member) phage antibody libraries from human V-gene repertoires. From such libraries, many different antibodies can be isolated against any antigen. The affinities of the phage antibodies are equivalent to affinities of antibodies produced during the secondary immune response, or to affinities of antibodies produced by hybridomas. The same technology can also be used to increase antibody affinity. The sequence of a binding phage antibody is mutated and higher affinity binders selected

from the mutant antibody library. Using this approach, the affinity of human phage antibodies have been increased more than 1000 fold, yielding human antibodies with $K_d < 2.0 \times 10^{-11}$ M (16).

5.1.3. Application of phage display to produce neutralizing antibodies to BoNT A (DAMD17-94-C-4034)

The overall goal of the project is to generate neutralizing monoclonal antibodies for Botulinum neurotoxins type A, B and E (BoNT/A, BoNT/B and BoNT/E). With funding from DAMD17-94-C-4034, we generated phage antibody libraries from mice immunized with BoNT/A H_C, from mice immunized with BoNT/A H_C and boosted with BoNT/A holotoxin and from humans immunized with pentavalent Botulinum toxoid. After selection on either BoNT/A H_C or Botulinum holotoxins, a panel of 51 murine and 79 human monoclonal antibodies to BoNT's were generated (Table 1). We focused on the further evaluation of the 92 monoclonal antibodies which bound BoNT/A. First, we epitope mapped these antibodies with respect to the domain of BoNT/A bound (binding domain (H_C), translocation domain (H_N) or catalytic domain (light chain) (Table 1).

Table 1. Specificity of BoNT binding scFv selected from phage antibody libraries.

scFv Specificity	Number of unique scFv				
	mice immunized with BoNT/A H_C, boosted with BoNT/A	mice immunized with BoNT/A H_C	Humans immunized with toxoid	Non-immune humans	Total scFv
BoNT/A H_C	10	18	6	10	44
BoNT/A H_N	2	0	4	2	8
BoNT/A light chain	21	0	16	3	40
BoNT/B	ND	ND	16	5	21
BoNT/C	ND	ND	6	5	11
BoNT/E	ND	ND	3	3	6
Total	33	18	51	28	130

Our efforts to identify neutralizing antibodies then focused on the H_C binding antibodies. Work of others had previously shown and verified that prevention of binding of toxin to receptor is the key step in preventing intoxication. The 44 H_C binding single chain Fv (scFv) antibodies were first epitope mapped using surface plasmon resonance in a BIAcore to identify the number of unique epitopes on H_C recognized (Tables 2 and 3, next page).

Antibodies binding 15 unique non-overlapping epitopes were identified (Tables 2 and 3). At least 1 scFv recognizing each epitope was further characterized with respect to binding constant and ability to neutralize BoNT/A in a hemidiaphragm study (Tables 2 and 3). The neutralization studies were done at USAMRICD in the laboratory of Dr. Desphande. This work identified that scFv binding 2 different epitopes on H_C had neutralizing capacity and that co-administration of the two antibodies (either C25 and S25 or C25 and 3D12) had an additive effect on toxin neutralization (Tables 2 and 3 and figures 1 and 2).

Table 2. Affinities, binding kinetics, and *in vitro* toxin neutralization results of scFv selected from phage antibody libraries.

scFv clone	Epitope	K_d[a] (M)	k_{on} (x 10^4 M^{-1} s^{-1})	k_{off} (¥ 10^{-3} s^{-1})	Paralysis Time[b]
S25	1	7.3×10^{-8}	1.1	0.82	85 ± 10[c]
C25	2	1.1×10^{-9}	30	0.33	151 ± 12[c]
C39	2	2.3×10^{-9}	14	0.32	139 ± 8.9[c]
1C6	3	2.0×10^{-8}	13	2.5	63 ± 3.3
1F3	4	1.2×10^{-8}	92	11	52 ± 1.4
C25 + S25 Combi					218 ± 22[c]
BoNT/A pure toxi					56 ± 3.8

[a] k_{on} and k_{off} were measured by surface plasmon resonance and K_d calculated as k_{off}/k_{on}.

[b] Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. Each value is the mean ± SEM of at least three observations.

[c] $p < 0.01$ with respect to BoNT/A., [d] $p < 0.05$ compared to C25.

Table 3. Affinities, binding kinetics, and in vitro toxin neutralization results of scFv selected from phage antibody libraries.

scFv clone	Epitope	K_d[a] (M)	k_{on} (x 10^5 M^{-1} s^{-1})	k_{off} (x 10^{-3} s^{-1})	Paralysis Time[b]
Immune Library					
3D12	4	3.69×10^{-8}	0.13	0.50	85 ± 5.0[c]
3F10	5	7.80×10^{-9}	0.80	0.62	55 ± 5.0
2B11	6	ND	ND	ND	ND
C25 + 3D12 Coml					179 ± 2.3[c]
BoNT/A pure toxi					63.9 ± 2.3
Non-immune Lib					
2A2	7	1.98×10^{-7}	2.35	46.7	56.3± 9.7
2B10	8	1.29×10^{-7}	5.57	71.6	62.3± 6.7
2E6	9	1.93×10^{-7}	1.19	23.0	60.9 ± 8.2
2H6	10	3.86×10^{-8}	2.20	8.50	63.0± 5.0
3G11	11	1.07×10^{-7}	0.83	8.88	58.4± 4.0
2A9	12	2.61×10^{-8}	0.25	0.66	71.0± 3.0
2B6	13	7.15×10^{-8}	1.09	7.80	61.9± 5.0
3F6	14	6.60×10^{-8}	4.69	30.9	60.4± 3.6
3C2	15	3.90×10^{-8}	2.10	82.0	61.9± 4.8

[a] k_{on} and k_{off} were measured by surface plasmon resonance and K_d calculated as k_{off}/k_{on}

[b] Time (min.) to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv + 20 pM BoNT/A, compared to time for BoNT/A alone. Each value is the mean ± SEM of at least three observations.

[c] $p<0.01$

ND: Not determined.

Figure 1. Evaluation of murine scFv neutralization of BoNT/A in a mouse hemidiaphragm model.

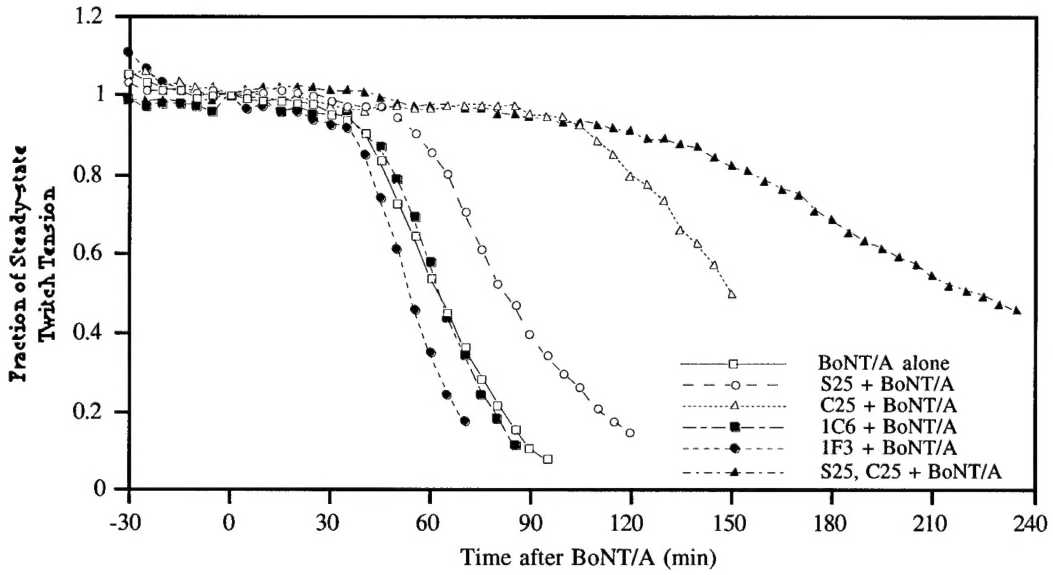
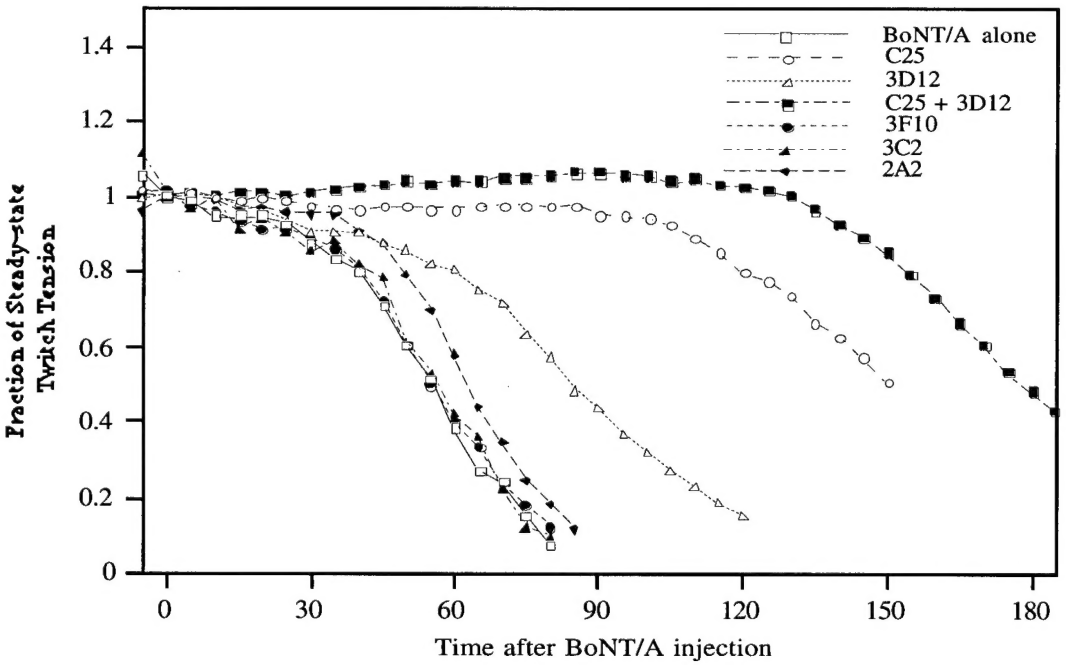


Figure 2. Evaluation of immune and non-immune human scFv neutralization of BoNT/A in a mouse hemidiaphragm model.



From the above results we concluded that:

1. After immunization of mice with BoNT/A holotoxin or humans with pentavalent toxoid, only a minority of monoclonal antibodies bind to the protective H_C. This

implies that for these immunogens, a large number of monoclonal antibodies must be studied to identify neutralizers.

2. Immunization of mice with BoNT/A HC yields a greater number of neutralizing antibodies.

3. Combinations of antibodies recognizing two different epitopes provide greater neutralization than use of a single antibody.

4. Dose-response studies indicate that higher scFv concentrations result in greater neutralization. This suggests that higher affinity antibodies will provide more potent toxin neutralization.

Based on these results, we hypothesized that human monoclonal antibodies capable of neutralizing BoNT/A, BoNT/B, and BoNT/E can be produced using phage display. For each serotype, it should be possible to achieve neutralization with either a single antibody, or a small (3 or less) number of antibodies. To achieve these results, we employed the technical objectives described below.

6. Body of Report

The body of the report is categorized according to the technical objectives in the Statement of Work.

6.1. Technical objective 1: Generate panels of monoclonal single chain Fv antibodies (scFv) that bind BoNT/A, B and E H_C.

6.1.1. Immunize mice transgenic (Xenomice) for the human Ig locus with BoNT/A, B and E H_C, harvest spleens, prepare RNA.

Fifteen mice were immunized with 10 ug of either BoNT/A, BoNT/B or BoNT/E H_C. Our previous work (and that of others) indicates that H_C is the most immunoprotective domain of BoNT's and thus our choice for immunizations. After three immunizations, mice had titers of 1:1000 to 1:10,000 for binding to the immunizing antigen. Spleens and lymph nodes were harvested from the mice. RNA was prepared from all 15 spleens and from the lymph nodes of 4 of the mice for each antigen.

High quality RNA was obtained from each mouse spleen and lymph nodes for each antigen (figure 7 left panel). Spleen and lymph node RNA was pooled for each antigen. 1st strand cDNA was synthesized from RNA and used as a template for amplification of V_H and V_κ genes. This amplification was successful for each antigen. The V_H and V_κ genes were spliced together to create scFv gene repertoires (figure 3, right panel). This splicing was also successful for each antigen.

6.1.2. Amplify V_H and V_L genes, prepare scFv gene repertories, and clone to create Xenomice scFv phage antibody libraries.

scFv gene repertoires from Xenomice immunized with BoNT/A, BoNT/B and BoNT/E H_C were gel purified and reamplified using primers containing appended NcoI (5' end of the gene) and NotI (3' end of the gene) restriction sites. A total of 20 0.5 ml PCR amplifications were performed for each antigen to ensure adequate DNA for library construction. The total yield of DNA was estimated to be 50 ug. Approximately 10 ug of the reamplified repertoires were digested with NcoI and NotI restriction

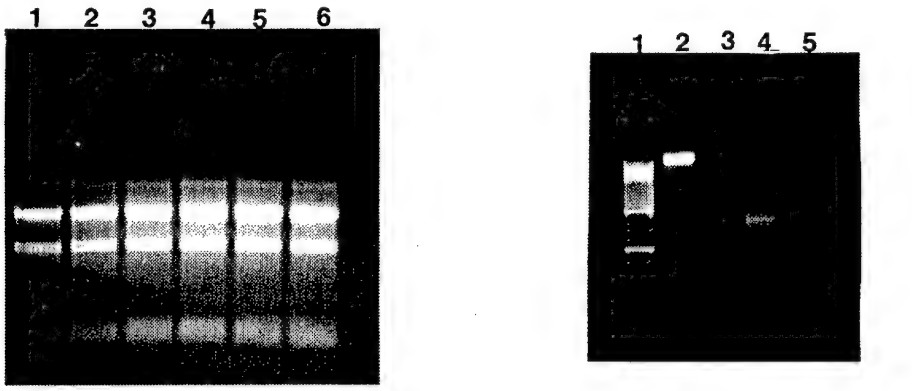


Figure 3. Results of RNA preparation and scFv gene repertoire generation. RNA was prepared from spleens (lanes 1, 3, 5)and lymph nodes (lanes 2, 4, 6) of mice immunized with BoNT/A (lanes 1 and 2), BoNT/B (lanes 3 and 4) or BoNT/E H_C (lanes 5 and 6) (left panel). The 28S and 18S ribosomal bands are clearly evident. V_H and V_k DNA was amplified from RNA and spliced together to obtain an scFv gene repertoire (right panel). Lane 1 = markers, lane 2 = digested vector DNA, lane 3 = purified scFv gene repertoire, lane 4 = spliced scFv repertoire before purification, lane 5 = negative control (V_H plus V_k without linker DNA).

enzymes and gel purified. After digestion and purification the yield was approximately 5 ug. pSYN2 phage display vector DNA was prepared using the CsCl method.

Approximately 100 ug of vector DNA was digested with NcoI and Not and the digested vector gel purified. Trial ligations were set up with varying ratios on scFv gene repertoire insert to vector DNA to identify the optimal ratio for ligation. Based on these results, ligations were scaled up to include 1 ug of insert DNA and 2 ug of vector DNA. Ligation reactions were extracted with phenol/chloroform and ethanol precipitated. Electrocompetent *E. coli* TG1 were prepared and 10 electroporations performed with each of the three ligation mixtures (BoNT/A, BoNT/B and BoNT/E) to generate phage antibody libraries. The electroporations were plated on TYE plates containing ampicillin and 1% glucose and yielding the following number of transformants (ampicillin resistance colonies, Table 4). The percentage of clones containing an scFv sized insert was determined by PCR amplification of colonies containing the plasmid with primers which flanked the scFv cloning site (Table 4). Vector background gave an insert size of 1.5 kb vs 0.7 kb for the scFv gene. Final library size was the product of number of ampicillin resistant colonies and the percentage of colonies with an scFv gene insert. For each of the immunogens, library size was at least 1 million clones.

Table 4. Phage antibody library size using scFv gene repertoires constructed from immunized Xenomice. Number of transformants = number of ampicillin resistance colonies. Corrected library size = (# of transformants) x (# of clones with scFv insert).

Immunogen	# of transformants	# (%) of clones with scFv insert	Corrected library size
BoNT/A H_C	3.7×10^6	12/19 (63)	2.3×10^6
BoNT/B H_C	2.9×10^6	13/17 (76)	2.2×10^6
BoNT/E H_C	3.4×10^6	15/16 (94)	3.2×10^6

To confirm library diversity, the PCR'ed scFv gene inserts from random colonies were digested with the frequently cutting restriction enzyme BstN1 (figure 4). For each

of the immunogens (A, B and E), the many different restriction patterns were observed, indicating that the library was diverse.

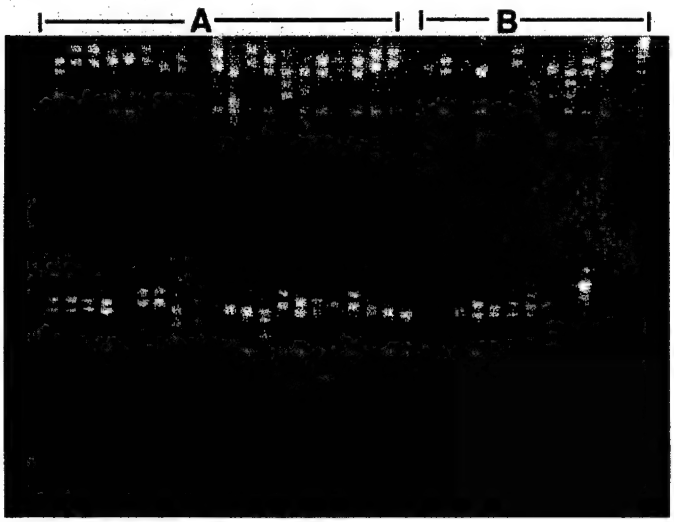


Figure 4. BstN1 restriction fragment analysis of scFv genes amplified from BoNT/A, BoNT/B and BoNT/E phage antibody libraries.

6.1.3. Begin selection of xenomice phage antibody libraries

To isolate anti-botulinum phage antibodies, the three Xenomice libraries described above were selected on immunotubes coated with either BoNT/A H_C, BoNT/B H_C, or BoNT/E H_C. Tubes were coated with 25 ug/ml of antigen (obtained from Ophidian Inc.). Phage were incubated for two hours, non-binding phage removed by multiple PBS washes, and specifically bound phage eluted by the addition of triethylamine. Eluted phage were used to infect E. coli TG1 and additional phage were prepared for the next round of selection. A second round of selection was then performed. The titers of eluted phage are reported in Table 5, below.

Table 5. Output titers of phage after the first and second round of selection on the indicated BoNT H_C serotype. Titer = number of ampicillin resistance colonies

Immunogen	Input phage titer	Output titer round one	Output titer round two
BoNT/A HC	Approx. 1.0×10^{12}	1.0×10^{4}	2.2×10^{8}
BoNT/B HC	Approx. 1.0×10^{12}	3.8×10^{4}	1.2×10^{7}
BoNT/E HC	Approx. 1.0×10^{12}	1.0×10^{4}	9.0×10^{8}

The results indicate a significant increase in titer between the first and second round, suggesting that enrichment for BoNT binding phage antibodies has occurred. To verify this, and identify monoclonal BoNT binding phage antibodies, individual colonies from the first and second rounds of selection were picked into 96 well microtitre plates and phage prepared by the addition of helper phage. After overnight growth, bacterial supernatant containing phage antibodies was used directly for a binding ELISA. For ELISA, microtitre plates were coated overnight with either either BoNT/A H_C, BoNT/B H_C, or BoNT/E H_C. Plates were washed and phage added. Binding of phage was detected with anti-M13 horse radish peroxidase. The results of the ELISA are listed below in Table 6.

Table 6. Results of ELISA analysis of 1st and 2nd round of selections Titer = number of ampicillin resistance colonies

Immunogen	Round One, # of positives	Round Two, # of positives
BoNT/A H_C	54/96	79/96
BoNT/B H_C	13/96	69/96
BoNT/E H_C	47/96	75/96

The results indicate the successful selection of monoclonal human phage antibodies to BoNT/A, BoNT/B, and BoNT/E H_C.

With respect to technical objective one, we are ahead of schedule, as we had predicted that library selection would not be completed until 4 months into fund year 2. Part of the reason for this is that we elected to concentrate on libraries generated from Xenomice, which permit immunization with the optimal immungen (H_C) and generate human antibodies.

6.2. Technical Objective 2: Develop a system for rapid production of scFv as fusion proteins with immunoglobulin Fc fragment.

We were able to get a jump on this technical objective using funds from the no cost extension of DAMD17-94-C-4034.

To determine the in vivo neutralization capacity of scFv, it was necessary to modify the molecules to prolong the serum half life. To do this, we decided to fuse the scFv to the Fc portion of human IgG1. The resulting scFv-Fc fusion would have a molecular mass of approximately 100 kDa, well above the renal threshold for clearance. The expression host we chose was the methyltrophic yeast *Pichia pastoris*. We chose *Pichia* due to its reported high expression levels of recombinant protein and the relative speed with which the fermentations could be performed (three to five days compared to several weeks for mammalian expression systems). Construction of scFv-Fc fusions also greatly reduced the time necessary to create the genetic constructs for expression. The scFv gene could be simply subcloned intact into the appropriate expression vector. Construction of complete IgG would require subcloning both the V_H and V_L genes separately. Pichia vectors for co-expression of two chains (as required for IgG) also do not exist, so we would have had to use a mammalian system. Since we wanted to analyze a relatively large number of scFv, it was decided to construct scFv-Fc fusions and use Pichia as the expression host.

6.2.1. Clone and express 3D12, C25. And S25 scFv as scFv-Fc fusions in Pichia pastoris

scFv-Fc fusions were constructed initially for the two murine scFv which showed neutralization capacity in the hemidiaphragm assay (S25 and C25) as well as for the human scFv 3D12 which also showed neutralization capacity in the same assay. Both C25 and S25 were expressed in shake flasks with yields after purification of 1.5 mg/L for C25 and 300 ug/L for S25

Next, we cloned the 3D12 scFv gene for expression as an scFv-Fc fusion in Pichia pastoris. Accordingly, 2 mg of C25 was purified on protein G for in vivo studies. In the case of S25 and 3D12, expression levels are only 250 ug/L. To generate adequate quantities of scFv-Fc for neutralization studies, we have developed fermentation capabilities using a 2L Applikon Bioreactor. 3D12 and S25 were fermented to a wet

weight of 350 - 400 g/L and 3 mg of 3D12 and 4 mg of S25 purified using protein G. Approximately 2 mg each of S25, C25 and 3D12 were sent to Ms. Terry Smith at USAMRIID to perform in vivo neutralization studies.

This technical objective was successfully completed.

6.3. Technical Objective 3: Confirm that monoclonal 3D12, C25, and S25 scFv-Fc fusions neutralize BoNT in vivo.

To determine neutralization capacity *in vivo*, mice were injected intraperitoneally either with 20 or 100 LD50s of BoNT/A alone or mixed with 50 ug of either C25 scFv-Fc or S25 scFv-Fc or a combination of C25 and S25 scFv-Fc fusions. The results are shown in figure 5. At the lower does of toxin (20 LD50s), there was significant prolongation of the time to death for each individual scFv-Fc fusion (right panel). Without antibody, all mice were dead by 24 hours. In contrast 6/6 mice receiving toxin plus S25 fusion were alive and 4/6 mice receiving C25 were alive at 24 hours.

Survival decreased with increasing time from antibody and toxin administration. At the higher dose of toxin (100 LD50s), only a minor prolongation is seen with either C25 or S25 scFv-Fc fusions compared to toxin alone (figure 5, right panel). When combined, the scFv-Fc fusions significantly prolong the time to death compared to when administered alone. Thus the in vivo results recapitulate the in vitro neutralization studies with respect to the additivity of monoclonals and toxin neutralization.

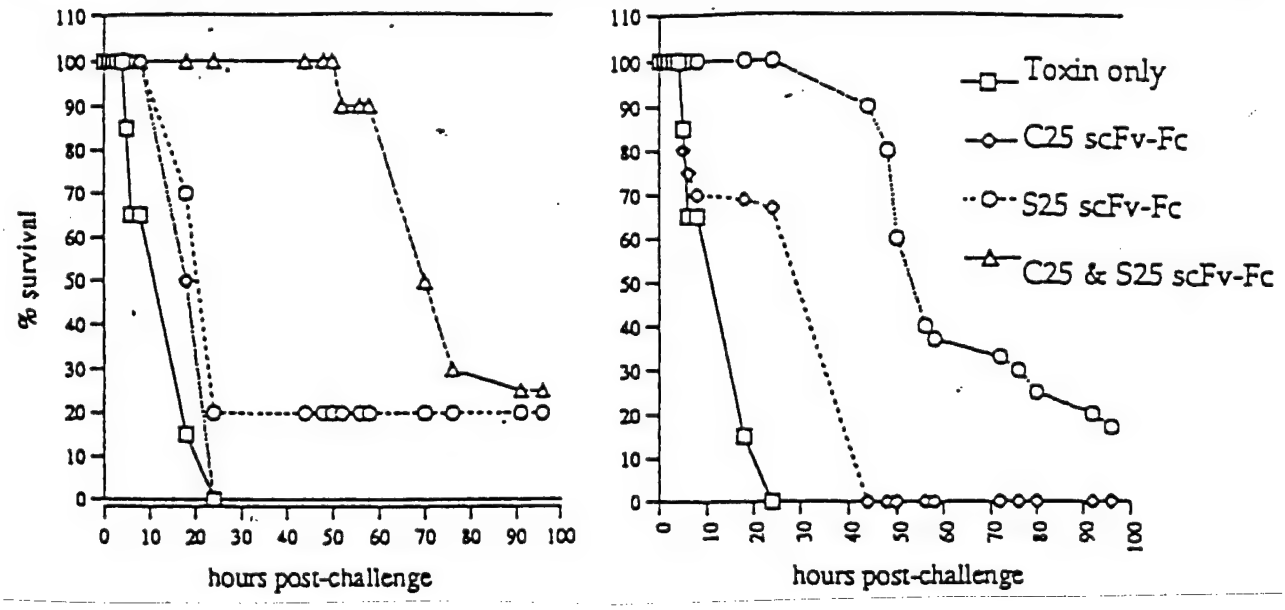


Figure 5. Results of in vivo toxin neutralization by C25 and S25 scFv-Fc fusions. Mice were injected intraperitoneally (i.p.) with 20 or 100 LD50s of toxin or toxin plus one or both of the scFv-Fc fusions. Right panel = 20LD50s of toxin, left panel = 100 LD50s of toxin.

The results described above showing additivity of toxin neutralization by a pair of monoclonal antibodies led us to hypothesize the existence of either two receptor 'binding sites' on H_C or a broad binding surface (also hypothesized by others). Neutralization would require blocking both sites. To test this hypothesis, we wanted to confirm the additivity of the two antibodies already studied which have an additive effect (C25 and S25). We also wanted to study an additional antibody which has in

vitro neutralizing capacity (3D12) which binds to the same epitope as S25. Thus in the next neutralization study, we planed to determine the neutralization capacity of 3D12 alone, C25+S25, C25 + 3D12 and S25 + 3D12.

To determine neutralization capacity *in vivo*, mice were injected intraperitoneally either with 20 or 100 LD50s of BoNT/A alone or mixed with 50 ug of either 3D12, or a combination of C25 and S25 scFv-Fc fusions, C25 and 3D12 scFv-Fc fusions or S25 and 3D12 scFv-Fc fusions. The results are shown in figure 6. At either does of toxin (20 or 100 LD50s), there was significant prolongation of the time to death with 3D12 scFv-Fc fusions (left panel). Without antibody, all mice were dead by 20 hours (20 LD50s) or 12 hours (100 LD50s). This is comparable to results observed for S25 or C25 scFv-Fc fusions (see results above). The combination of C25 and S25 gave similar results to those previously observed (prolongation of time to death, with 30% of mice surviving) (right panel). For 3D12 and C25 combined, 70% of mice survived 100 LD50s. To our surprise, all mice receiving the combination of 3D12 and S25 survived 100 LD50s.

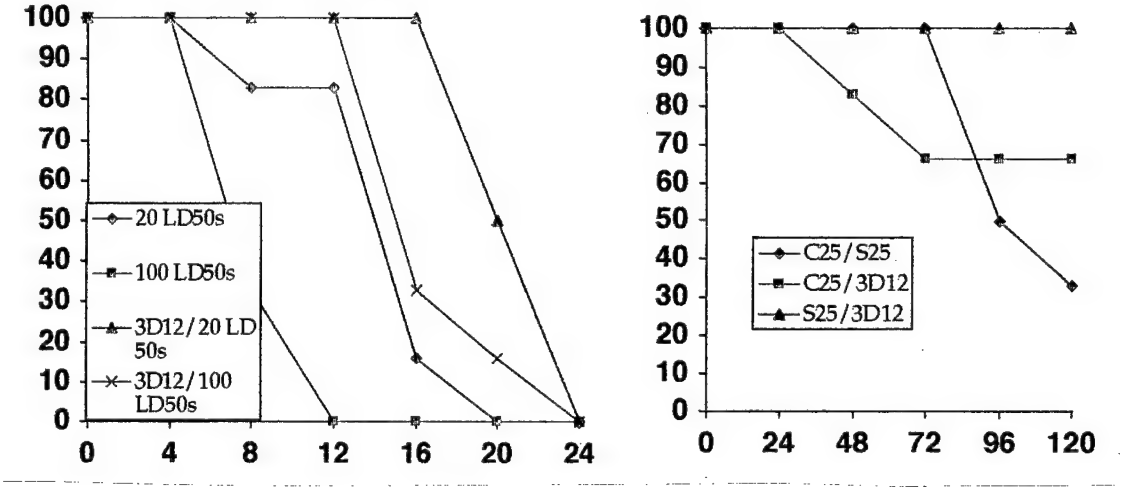


Figure 6. Results of in vivo toxin neutralization by 3D12, C25 and S25 scFv-Fc fusions. Mice were injected intraperitoneally (i.p.) with 20 or 100 LD50s of toxin or toxin plus one or both of the scFv-Fc fusions. Left panel = toxin controls and 3D12 scFv-Fc fusion. Right panel = 100 LD50s of toxin plus pairs of scFv-Fc fusions.

This result was surprising because our earlier studies with epitope mapping suggested that 3D12 and S25 recognize largely overlapping epitopes. This epitope mapping was performed using surface plasmon resonance in a BIAcore where the amount of binding was determined for either antibody alone and compared to the amount bound when the antibodies were co-injected. This type of analysis does not actually determine whether both antibodies can bind toxin at the same time. We therefore repeated the epitope mapping of 3D12, C25 and S25, by immobilizing the 3D12 scFv-Fc fusion on a BIAcore chip. We then injected BoNT/A H_C and observed it bind (see figure 7 below). After BoNT/A H_C was bound, we then injected either 3D12 (as a negative control) S25 or C25 scFv-Fc fusions. The results (figure 7) indicate that when BoNT/A H_C is bound by 3D12, either S25 or C25 can also bind. This demonstrates that each of these antibodies recognize non-overlapping epitopes. The *in vivo* toxin neutralization results confirm the additivity of toxin neutralization by pairs of monoclonal antibodies and suggest two separate receptor binding sites both of which must be blocked for potent toxin neutralization. For the first time, we observed

complete protection of mice from 100 LD50s with the combination of S25 and 3D12 scFv-Fc fusions.

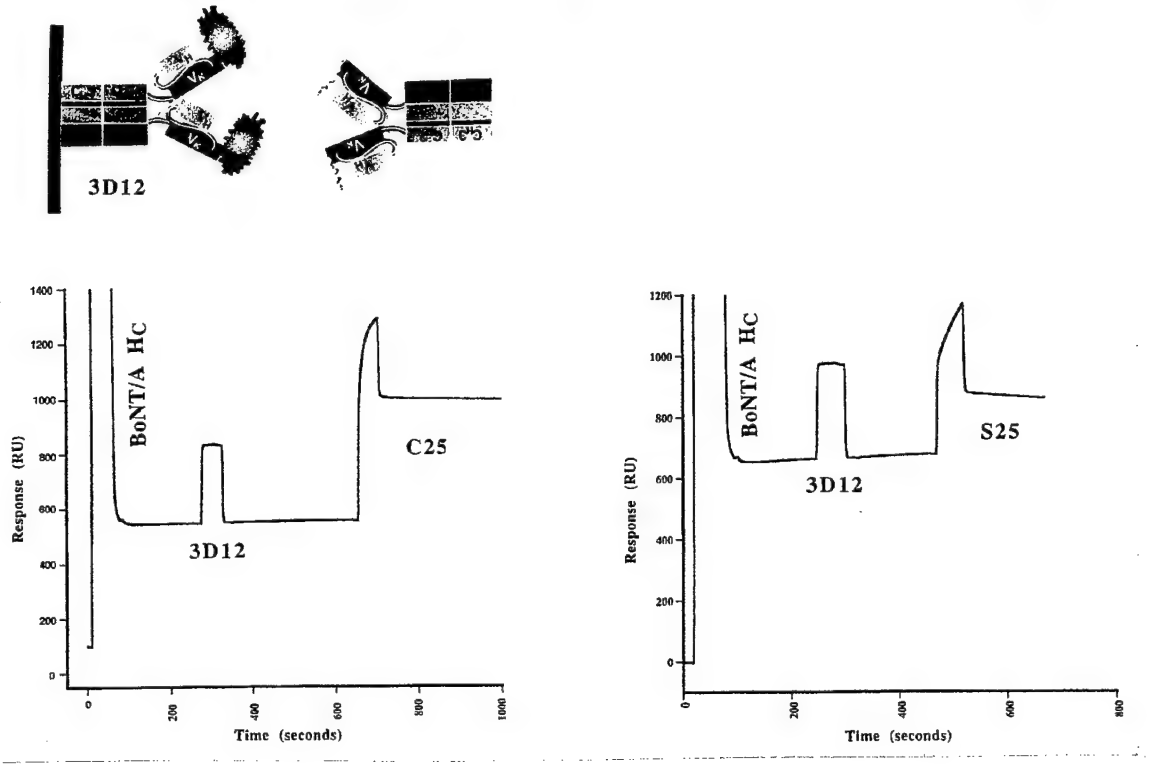


Figure 7. Epitope mapping of 3D12, C25 and S25 scFv-Fc fusions using BIAcore. 3D12 scFv-Fc fusion was covalently coupled to the chip surface (upper cartoon). BoNT/A H_C was injected and approximately 500-600 RU bound. Injection of 3D12 (negative control) showed no binding, while either C25 or S25 could bind.

To confirm the toxin neutralization of the 3D12 and S25 scFv-Fc fusion combination and define the upper limit of toxin neutralization. Thus we re-expressed S25 and 3D12 scFv-Fc fusions from P. pastoris, purified then by Protein G affinity chromatography and gel filtration and confirmed their immunoreactivity both by ELISA and by BIAcore analysis. Both these assays confirmed that the scFv-Fc fusions were active. Purified scFv-Fc fusion protein was delivered to Ms. Theresa Smith at USAMRIID for in vivo neutralization studies in mice. The ability of an equimolar mixture of S25 and 3D12 scFv-Fc fusion protein to neutralize 100 mouse LD50s was confirmed, with 2/3 of the mice completely protected against death from toxin injection and the remainder of the mice showing a significant delay in time to death (figure 8). At 1000 LD50s, there was an approximately two fold delay to time of death of the mice.

To examine potential mechanisms for the failure of complete protection of single scFv-Fc, and the delay but not prevention of death with multiple scFv-Fc used at higher LD50s, we determined the pharmacokinetics of scFv-Fc fusions in mice.

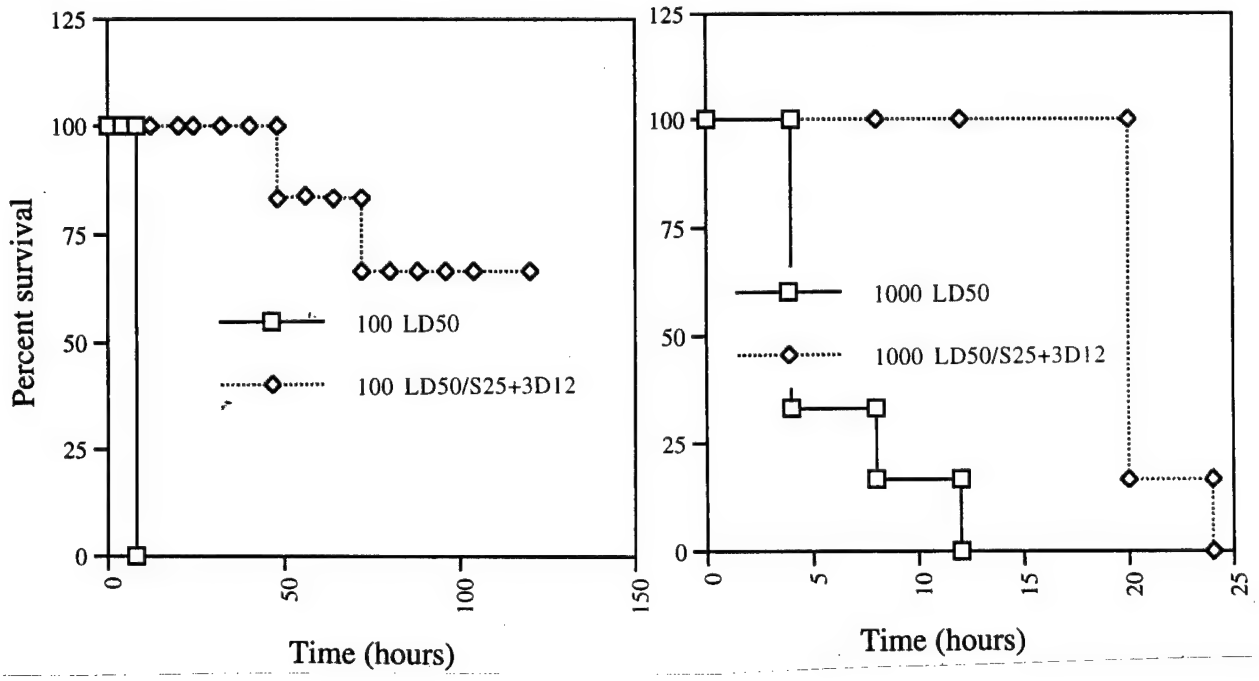


Figure 8. Results of in vivo toxin neutralization by 3D12 and S25 scFv-Fc fusions. Mice were injected intraperitoneally (i.p.) with 100 or 1000 LD50s of toxin or toxin plus a mixture of the two scFv-Fc fusions. Left panel = 100 LD50s. Right panel = 1000 LD50s.

To determine the pharmacokinetics of scFv-Fc fusions in mice, the C25-Fc fusion protein was radiolabelled and administered to mice in 20 ug doses, both intravenously and intraperitoneally. Figure 9 shows that the C25-Fc fusion had dramatically prolonged serum perseverance whether administered intravenously or intraperitoneally, with a $t_{1/2}$ for the beta phase of 52 and 93 hours for i.v. or i.p. administration respectively. This compares to only 2.5 to 3.5 hours for the scFv. The increased retention of the scFv-Fc fusions can be attributed to the increased size of the scFv-Fc homodimer which places the mass above the renal threshold. However, the serum level maintained over time by the scFv-Fc fusions is only 20-25% of that achieved by IgG. This is related to the pattern of *Pichia pastoris* glycosylation, which leaves high mannose which can be rapidly cleared by mannose receptors resulting in a decreased serum level. This marked decrease in half life could explain failure to observe more potent toxin neutralization.

6.3.1. Construction of additional Pichia pastoris expression vectors

While not one of original technical objectives, the results above indicated a need to attempt to alter the Pichia expression vector to eliminate Fc glycosylation in order to increase the serum half-life. Thus, we attempted to increase the serum level by removing the single glycosylation site on the human Fc by site directed mutagenesis. At the same time, we wanted to engineer the leader sequence of the Pichia expression vector so that we can directly subclone the scFv gene for expression as an Fc fusion directly from the phage display vector rather than use PCR. This eliminates the need to sequence after subcloning and should save considerable time. First, we removed an NcoI site from the pPIg1 vector backbone and introduced an NcoI site into the leader sequence using site directed mutagenesis to create pPIg2. A correct clone was identified by restriction analysis followed by DNA sequencing. We then removed the single N-

linked glycosylation site from the Fc part of the molecule. Again, a correct clone was identified by DNA sequencing.

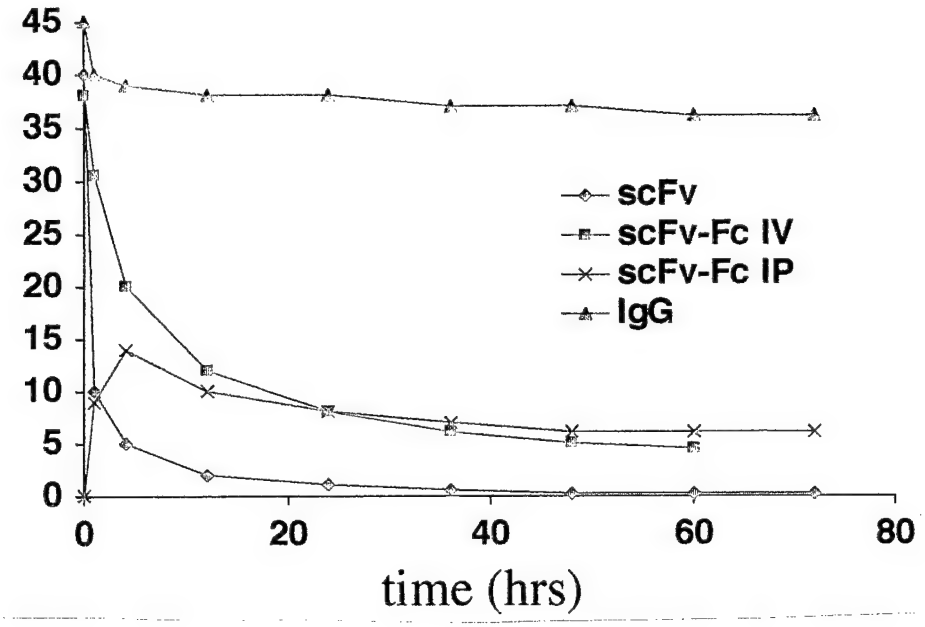


Figure 9. Biodistribution of scFv, scFv-Fc fusions and IgG injected into mice either intravenously or intraperitoneally.

The C25 scFv gene was subcloned into pPIg3, the engineered Pichia pastoris expression vector containing Nco1-Not1 restriction sites for scFv subcloning and with the Fc glycosylation site removed by site directed mutagenesis. A clone containing the correct C25 scFv gene insert was identified by DNA sequencing. C25 scFv-Fc fusion was expressed and purified by Protein G affinity chromatography followed by gel filtration. SDS-PAGE indicated a single band of the appropriate size. Successful elimination of glycosylation was confirmed by digesting purified scFv-Fc fusion with PNGase F. SDS-PAGE indicated ni difference in the size of the band before and after treatment with PNGase F. In contrast, treatment of purified C25 scFv-Fc fusion where the gylcoyslation site had not been removed showed a significant decrease in size by SDS-PAGE after treatment with PNGase F. We interpret these results to indicate successful removal of Fc glycosylation. Purified fusion protein was shipped to Dr. Greg Adams at Fox Chase Cancer Center for *in vivo* determination of pharmacokinetics.

6.4. Technical Objective 4: Determine the relative importance of affinity in achieving high titer BoNT neutralization

Beginning this objective was contingent on identifying and confirming the neutralization efficacy of a single monoclonal antibody. Since no potently neutralizing single monoclonal was identified, this objective was deferred.

7. Key Research Accomplishments

7.1 Immunization of Xenomice with BoNT/A, B, and E H_C

7.2 Successful RNA preparation from Xenomice immunized with BoNT/A, B, and E H_C.

7.3 Construction of phage antibody libraries from Xenomice immunized with BoNT/A, B, and E H_C.

7.4 Isolation of a panel of human monoclonal antibody fragments from phage libraries constructed from Xenomice immunized with BoNT/A, B, and E H_C.

7.5 Construction of a Pichia expression vector for expression of scFv-Fc fusions.

7.6 Expression and purification of two murine (C25 and S25) and one human (3D12) scFv-Fc fusions.

7.7 Demonstration that C25, S25 and 3D12 scFv-Fc exhibit in vivo toxin neutralization and that a combination of two of the scFv-Fc fusions can neutralize 100 toxin LD50s.

7.8 Demonstration that scFv-Fc fusions have significantly longer in vivo half lives than scFv, but that Fc glycosylation results in the Fc having a significantly shorter half life than IgG.

7.9 Construction of a scFv-Fc expression vector where the Fc glycosylation site is removed.

8. Reportable Outcomes

8.1 Accepted manuscript:

Powers DB, Amersdorfer P, Poul M-A, Shalaby MR and Marks JD. Expression and characterization of single-chain Fv-Fc fusions in Pichia pastoris. J. Imm Meth. In press.

8.2 Patent Application

Marks JD and Amersdorfer P. Therapeutic monoclonal antibodies that neutralize botulinum neurotoxins.

8.3 Degrees Obtained

Peter Amersdorfer, Ph.D.

9. Conclusions and Future Work

During the year we have made considerable progress towards our goal of generating human monoclonal antibodies which can neutralize Botulinum neurotoxins A, B, and E. Progress has proceeded on two fronts: 1) generation of a large panel of human antibodies from mice transgenic for the human Ig locus immunized with toxin H_C and 2) demonstration that scFv phage antibodies previously generated from phage libraries can successfully neutralize toxin.

On front one, we have achieved first year technical objectives 1, with successful construction and selection of phage antibody libraries made from mice immunized with A, B, and E H_C. It is anticipated that in fund year 2, selections will be completed and the binding clones characterized with respect to affinity, epitope recognized, toxin specificity, and DNA sequence. This will give us a large panel of human antibodies to the three toxins which can be evaluated in year three for toxin neutralization.

On front two, we achieved technical objectives 2 and 3. A Pichia expression vector was constructed which permitted successful expression and purification of scFv-Fc fusions and these fusions had a significantly longer in vivo half life than scFv. The

prolonged half life allowed the demonstration that the three scFv which neutralize toxin in vitro neutralize toxin in vivo. Moreover, a combination of two antibodies recognizing separate toxin epitopes leads to more potent toxin neutralization. This result confirms our in vitro work and suggests two toxin binding sites on receptor(s). A study of in vivo pharmacokinetics indicates that glycosylation of the Fc leads to a significantly shorter in vivo half life than IgG. Presumably increasing the half life will lead to more potent toxin neutralization. We therefore removed the glycosylation site by site directed mutagenesis. In the coming year, we will evaluate the in vivo pharmacokinetics of the new scFv-Fc fusion. If the half life has not been increased to that of an IgG, we plan on constructing complete IgG from the scFv genes for more accurate in vivo studies and determination of toxin neutralization.

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